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14. ABSTRACT

Tissue nitration due to environmental heating (EH) may be tied to availability of NOx in the lungs. Hemorrhagic mimics of EH/MMW (millimeter wave) blood pressure drops showed no increase in NOx levels. Nitration levels did spike as temperature increased with MMW exposure. Infrared heating could not be matched to MMW heating profiles. Histological examination did reveal a pronounced response 35GHz exposure at long continuous exposure. Edema, blood vessel engorgement, panniculus muscle changes, adipose tissue, and epithelial stripping are some of the observed changes. Immunochemistry revealed mixed results. Of the cytokines investigated only IL-1b provoked a response at 38C. Various nitrated proteins were elevated in MMW exposed post-recovery animals. Plasma taken from exposed animals and put on cultured macrophages did not evoke responses greater than an LPS challenge. Proteomics of plasma and genomics of tissue from MMW exposed animals have provided some suggestions as to possible biomarkers. Myeloid-related, matrix metalloproteinases, and Acute Phase protein are representative. Animals taken to death with continuous exposure to 35 and 94 GHz at power densities of from 60 to 100 mW/cm2 suggest that calibrating on the core body temperature is the best way to adjust exposures.

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6. List of manuscripts submitted/published since last progress report:
a. Kalns JE, Ryan KL, Mason PA, Scruggs JP, Gooden R, Kiel JL. 3-Nitrotyrosine is found in liver and lung following sub-lethal whole body heating and hemorrhagic shock in rats. Manuscript submitted to nitric oxide.
b. Kalns, J.E., P. Mason, L. Soza, W. Lawrence, J. Scruggs, J. Kiel, R. Blystone, and K. Ryan. Environmental heat induced 3-nitrotyrosine increase in lung and liver. In preparation.
c. Ryan, K., P. Mason, L. Soza, W. Lawrence, J. Scruggs, J. Kiel, R. Blystone, and J. E. Kalns. Blood loss causes nitric oxide production but not 3-nitrotyrosine accumulation in tissue. In preparation.

7. Scientific personnel supported by this grant along with their percent effort:
a) Robert V. Blystone - P.I. - 100% summer, 20% academic year
b) Maureen McConnell – Research Technician – 100%
c) Laura Soza – Research Technician – 100%
d) William Lawrence – Research Technician – 100%
e) Beth Brewer – Research Technician – 100%
f) Maureen McConnell – Research Technician – 100%
g) Erica Gutierrez – Undergraduate Research Assistant – 100% summer, 20% academic year
h) Sylvia Stewart – Part-time secretary – 10%

8. Inventions/Patents/Discoveries: None

9. Collaborators/Consultants
a) Dr. Johnathan Kiel, Senior Scientist, AFRL/HEDB, Brooks City Base, Texas. Dr. Kiel heads a research group that investigates the cellular responses to 35 and 94 GHz, which directly supports and compliments our efforts at the animal level. Both CoPI's are members of Dr. Kiel's research team. Our research efforts are tightly coordinated.
b) Dr. Patrick Mason – Research Physiologist, AFRL, HEDR, Brooks City Base, Texas. Dr.

Mason serves as liaison of HEDR to our group. He provides support in terms of exposure equipment, lab space, animal facilities, and office space. He participates in our research meetings and keeps us mindful of the Air Force mission.
c) Col. Jeffrey S. Eggers, Chief, Comparative Pathology, AFRL/HEDV, Brooks City Base. Dr. Eggers provides pathology support for our efforts. He greatly contributes to our research discussions.
d) Dr. Frank A. Witzmann, Prof. Of Cellular & Integrative Physiology, School of Medicine, Indiana University, Indianapolis, IN. Dr. Witzmann analyzed rat macrophage nitrated proteins activated after exposure to MMW and EH in order to determine the effect this plasma on the activation status of rat macrophages.
e) Dr. John M. Frazier, Senior Scientist, AFRL/HEST, Wright-Patterson Air Force Base, Ohio. Dr. Frazier's lab is performing genomic readouts of prepared tissue samples from our exposed animals in order to identify potential biomarker candidates.

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your personnel while being supported by AFOSR: None

11. Key Findings/Results/Accomplishments:

History

Dr. Melvin R. Frei demonstrated experimental animals would go into shock and die with exposure to millimeter wave (MMW) energy. (Frei *et al.,* 1995) Several of his collaborators, Dr. Kathy Ryan and Dr. Patrick Mason extended and continued his work. (Kalns *et al.,* 2000) The lab of Dr. Johnathan Kiel was dealing with issues of MMW exposure at the cellular level. Kiel and his collaborator, Dr. John Kalns, joined with Ryan and Mason in developing the research proposal whose progress is now being summarized. The work outlined in this proposal was to complement at the whole animal level the cellular work being performed in Dr. Kiel's lab. Dr. Blystone joined the project to provide input of an anatomical nature and to coordinate the activities of all parties.

The commercial and military use of MMW is increasing, especially at the 35 Gigahertz (GHz) and 94 GHz frequencies. The biological effects of radiofrequency radiation (RFR) at these wavelengths are poorly understood. Our research group has revealed that protein nitration is occurring in animals exposed to MMW for prolonged periods. (Kalns *et al.,* in preparation) The premise of our research proposal was to identify biomarkers of MMW exposure; and as a tool for that identification, protein nitration was a logical place to start. The objectives listed above represented our thinking in 1999 as to a path to identify a biomarker(s) of MMW RFR exposure.

Time Table of Activity

A timetable indicating milestone events by the BioMarkers research group is found below.

February 2000	the grant is funded
March	the first technician is hired
May	LPS exposure experiments underway; various molecular tests initiated
June	Animal protocol approved; physiology recording software in place; LPS experiments continue
July	CRADA approved; first LPS pathology available
August	Environmental Heat (EH) experiments started; Michelson conference

September	second technician in place; genomic and proteomic work suggested; anesthesia discussions
October	third technician in place; duplex anesthesia explored; telemetry "cut-down" effects on genomic discussed
November	EH work well underway, anesthetic problems noted
December	Labview reworked for physiology data collection; hemorrhage study well underway
January 2001	Tissue processing for proteomics; experience with MMW generator gained
SECOND YEAR	
February	the "snout" EH experiment developed; tissue work for EH underway; hemorrhagic study still in progress; LNAME research readied for FASEB; cell culture flask work underway
March	analysis of EH data; analysis of hemorrhagic data; LNAME research control data problems
April	FASEB; first MMW data; redefined technician support for grant period; cell culture dosimetry work
May	"snout" data analysis; anesthesia effects on MMW; LNAME analysis
June	MMW transmitter problems; histology reveals vascular effects in MMW skin; IR explored as positive control
July	MMW transmitter down; 94 GHz work planned; first proteomic data; chronic v acute MMW discussed
August	MMW transmitter moved; IR work underway with methodology problems; proteomics suggest acute phase response; thermal heating models v MMW heating models developed; funding for Post-doc available; breath markers discussed for first-time
September	follow-on grant developed; MMW cytokine data ambivalent; power levels for MMW explored; exposure recovery work in progress
October	MMW and IR results suggest skin focus; first genomic material in preparation; focus on grant
November	EH and MMW data analysis suggest different temperature response
December	No-cost extension filed; temperature twin model in doubt (IR contributes minimally); genomic data in analysis; time to death (TTD) experiments developed; follow-on grant proposal submitted
January 2002	MMW Transmitter down; four BEMS abstracts in preparation; more genomic samples sent; additional LNAME samples needed; TTD underway with three temp samples sites and five variables; Veridian post-doc search stalled
EXTENSION PERIOD	
February	TTD experiments results indicate two trends; animal positioning reexamined
March	94 GHz work well underway; hematocrit data correlation to mean arterial pressure (MAP); subcutaneous data reappraised
April	breath experiment outlined; LNAME add-on work completed; TTD completed, awaiting the statistics; post-doc search still stalled

Transmitter Calibration

The RFR transmitter has presented our research group with some interesting challenges. Each time the transmitter settings are altered between 35 ad 94 GHz, it is must be recalibrated. (Please

note that the transmitter is shared between several research groups.) The power density settings varied from about 50 mW per cm^2 to about 100 mW per cm^2. As our sample size of MMW-treated animals increased, we explored power density and frequency more thoroughly. As dosimetry was performed to calibrate the instrument, we discovered the accuracy for a specific power density setting was ± 15 mW per cm^2, a relatively wide range. The "dosing" of the transmitter is as much an art as a science. We are fortunate to have three experienced technicians at Brooks with long-term experience at calibrating the RFR transmitter. As a precaution, each time the transmitter is "dosed", we must run several test animals to verify the new settings.

Anesthetic Modification

Another unanticipated challenge has been the choice of anesthetic. The original research reported seven years ago was based on the use of ketamine or, less often, pentobarbital and urethane. Most subsequent research continued the use of ketamine. As we performed our experiments, we noted animal discomfort during surgery and exposure. We finally employed a duplex anesthesia consisting of ketamine and xylazine. Also as evolving research protocols suggested, we performed more recovery-based work (following animals after exposure for up to 72 hours). The physiological response of the exposed animals varied depending on the anesthesia type. Ryan had recorded earlier that MMW exposed rats died at lower colonic temperatures than experienced with other species. One reason for the development of Objective Three of this research had to do with this core temperature difference at death. Using the duplex anesthesia, the end point core temperature of rats resembled that of other species

Addition of Genomics and Proteomics

A major shift in our thinking had to do with the added possibility of genomic and proteomic (G/P) analysis. With additional resources made available in October of 2000, we began modifying our research approach to take advantage of these molecular tools. Post-exposure recovery of the animals is important for G/P sample taking. Most previous work dealt with animals sacrificed while still under anesthesia associated with exposure. Given the sensitivity of the molecular techniques, we had to develop a methodology that would minimize tissue and animal trauma. For example, post-surgical animals could not be given ibuprofen for fear that it might alter the genomic profile. Each step of our animal handling was re-examined. The addition of the G/P work placed time pressures for the attainment of the originally outlined five objectives. When we discovered that by using duplex anesthetic, species differences in exposure to death endpoint were less obvious; we decided not to explore the species-based objective three as originally outlined in the proposal. The time and budget gained were used to support the new G/P effort.

Addition of Possible Infrared Control

It is difficult to define a control for the MMW exposure. EH exposures provided a comparison for MMW effects. As various people reviewed our work, it was suggested that infrared exposures could serve as a form of control for our MMW work. We explored this possibility and conceived of the idea of a temperature twin. However, we discovered that IR caused heat movement from the outside in and MMW caused heat movement from the inside out. We determined that Infrared would not be a suitable temperature twin.

Nitration - Environmental Heating Exposure

Tissue response to environmental heating was examined by measuring protein nitration (NO_2) and NOx production (see Figures 1 and 2).

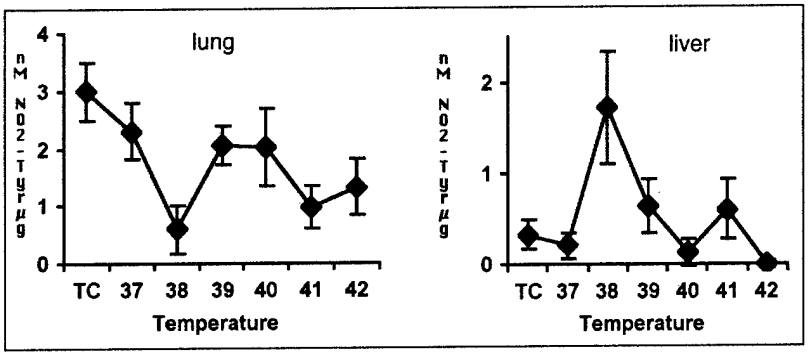


Figure 1: Nitration in lung (left) and liver (right) during exposure to environmental heating (42°C ambient temperature). ELISA was used to measure 3-nitrotyrosine (3-NT) in tissue lysate incorporating 3-NT antibody (n = 6/group).

The data revealed a drop in the lung NO_2 at 38°C and a spike in liver NO_2 at the same temperature. In these initial environmental heating experiments, the entire animal was placed inside the heated chamber (42°C) resulting in the animal breathing the warm air. After evaluating the above data, the placement of the animal inside the chamber was modified so that the animal would breath room temperature (23–24°C) air. Breathing room temperature air raised the nitration response by about 0.5 nM in lung and dropped the liver response by about 0.5 nM. Figure 2 shows NOx concentrations in liver and lung. These concentrations decreased below initial temperature control (TC) values as core temperature increased.

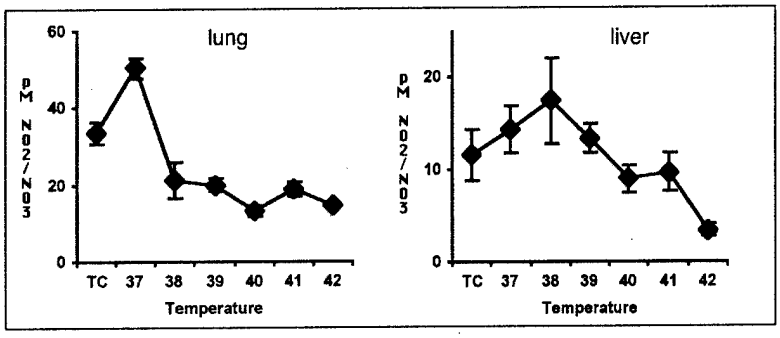


Figure 2: NOx in lung (left) and liver (right) during exposure to environmental heat (42°C ambient temperature). Levels measured by enzymatic/flurometric assay.

A hypothesis for the difference in the response patterns of NO_2 and NOx is that there is a high baseline concentration of NO_2 in the lungs and with a slight temperature increase the lungs begin to release NO_2 resulting in a subsequent increase in the NOx concentration in the lungs. Thus, the availability of NO may determine the overall abundance of nitrated proteins.

Nitration - Hemorrhage Study

Rats were bled to a mean arterial pressure (MAP) of 75 mm Hg in an effort to mimic hemorrhagic shock that was induced by either prolonged MMW or environmental heat exposure. The animals were kept at room temperature (24–25°C) during the experiment. Figure 3 shows that a reduction in MAP by removing blood volume has virtually no effect on protein nitration in either lung or liver.

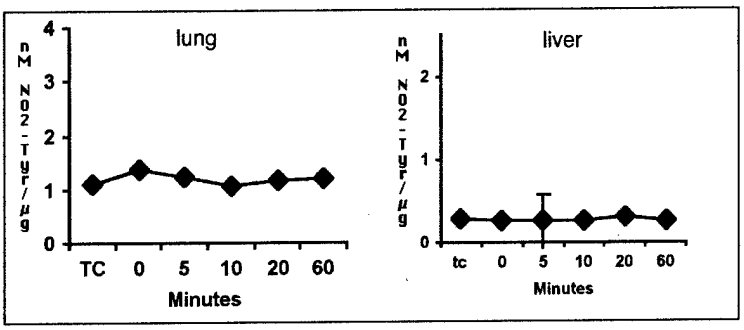


Figure 3: Nitration in lung (left) and liver (right) following hemorrhaging each animal to a MAP of 75 mm Hg. ELISA was used to measure 3-nitrotyrosine (3-NT) in tissue lysate incorporating 3-NT antibody.

The NOx data revealed a different response than NO_2 in hemorrhagic animals. Lung and liver showed an increase in NOx levels ten minutes after the MAP was reduced to 75 mm Hg (see Figure 4).

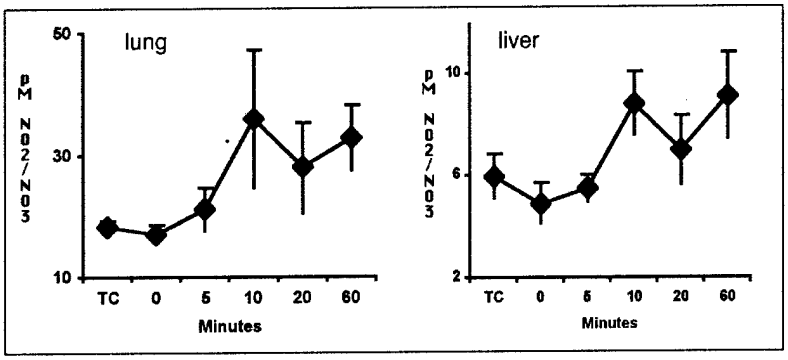


Figure 4: NOx in lung (left) and liver (right) in animals whose MAP was reduced to 75 mm Hg and blood samples collected at indicated time intervals. NOx levels were measured by enzymatic/flurometric assay.

Nitration - MMW Study

Nitration levels in MMW-exposed animals revealed a substantial increase in lung at 38°C (see Figure 5). This pattern is very different from that resulting from exposure to environmental heat (see Figure 2). The liver nitration response in MMW-exposed animals has a dip at 38°C. Comparison of these MMW and environmental heat data reveal more similarity to one another than does the lung data. However, both the MMW- and environmental heat-exposed animals showed changes in nitration patterns with heating, whereas, as indicated in Figure 3, there were no changes in nitration levels in the hemorrhaged animals.

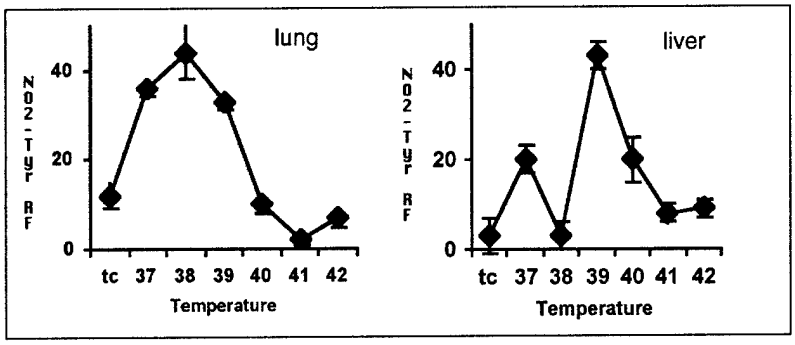


Figure 5: Nitration in lung (left) and liver (right) during 35-GHz exposure. 3-NT antibody in conjunction with confocal microscopy was used to measure 3-nitrotyrosine levels.

An interesting finding was a continuous increase in the amount of NO_2 (as measured using 3-nitrotyrosine antibody using flow cytometry) in blood leukocytes during MMW exposure. The lung, liver, and blood results are consistent with our hypothesis that nitration is a dynamic

process and that the observed changes occur very rapidly and the direction of change is highly dependent upon the organ.

Infrared Exposure

There is always the question as to which stressor is the best thermal control for MMW exposure. Comments could be made that environmental heat raises the core and skin surface temperature to match MMW exposure, but the dermis and subcutaneous temperatures are low. Another thermal control could be infrared heating. Figure 6 shows the results of using only infrared heat (1100 nm peak spectral irradiance) to match the skin and subcutaneous temperatures during prolonged MMW exposure. Infrared heating produced little or no change in the colonic temperature. Based on these data showing no substantial change in colonic temperature, we do not believe that infrared heating alone is a good thermal control for prolonged MMW exposure. However, we hypothesize that infrared heat combined with environmental heat would be a good thermal control for prolonged MMW exposure. Furthermore, the lack of an increase in colonic temperature during infrared heating makes infrared heat exposure a good thermal control for the proposed brief MMW exposures during which there is no colonic temperature increase.

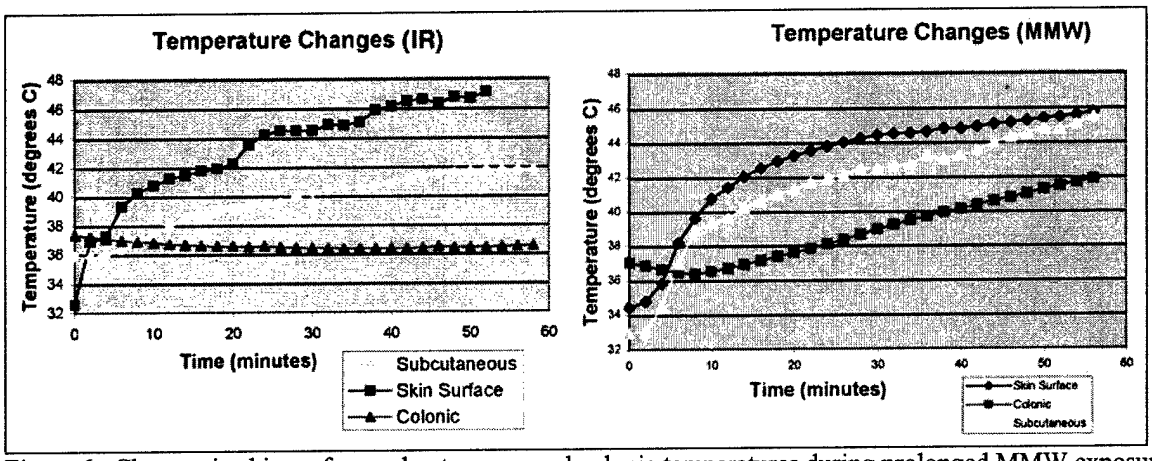


Figure 6: Changes in skin surface, subcutaneous, and colonic temperatures during prolonged MMW exposure or infrared heating.

Comparative Histology

The skin is a stratified structure and the most obvious layers are epidermis (about 30 μm thick; area 4 on Figure 7) and dermis (very approximately 1 mm thick and seen in areas 1, 2, and 3). The dermis is itself stratified with an area containing hair follicles and shafts (see area 2), adipose cells mixed with blood vessels (area 2) and a layer of striated muscle (area 1). The muscle has been given a variety of names including panniculus. Below the muscle layer is another layer of adipose and collagen. The muscle contains various nerve bundles and endings.

In our experiments, skin and muscle pathology samples were taken from the exposed and unexposed sides of the rat. Sections were examined using standard H&E, proliferating cell nuclear antigen (to detect recent DNA synthesis cycle), iNOS (to identify immune competent cells), 3-NT (to detect oxidative stress), and caspase 3 (to detect apoptosis in gut) stains.

Images presented in Figure 7 compare sham and 35-GHz exposed rats. These exposures amounted to approximately 270 joules administered to the lower left abdominal region over 53

minutes at 90 mW/cm^2. These rats were euthanized at 0, 24, or 72 hour post-exposure. The skin was taken from an anatomical location at which the distance between the skin surface and the muscle layer is about 0.5 mm (see sham). At 0 hours post-prolonged MMW exposure, this distance increased to 0.85 mm. Contributing to the swelling in this area is massive extravasation of blood cells (see asterisk). The blood vessels in this area are clearly engorged as seen at 24 hour recovery although the tissue swelling has diminished. The blood vessels continued their enlarged state at 72 hour but with some resolution.

Distinct changes in the striated muscle layer are apparent in successive recovery time samples. Sham muscle appears "plump" with little space between the muscle cells. With successive recovery periods, the muscle cells contract, lose their striation pattern, stain darker, and develop a halo round each cell. These changes are characteristic of successive stages of cell death. The adipose layer changes with the cells enlarging or sometimes apparently rupturing.

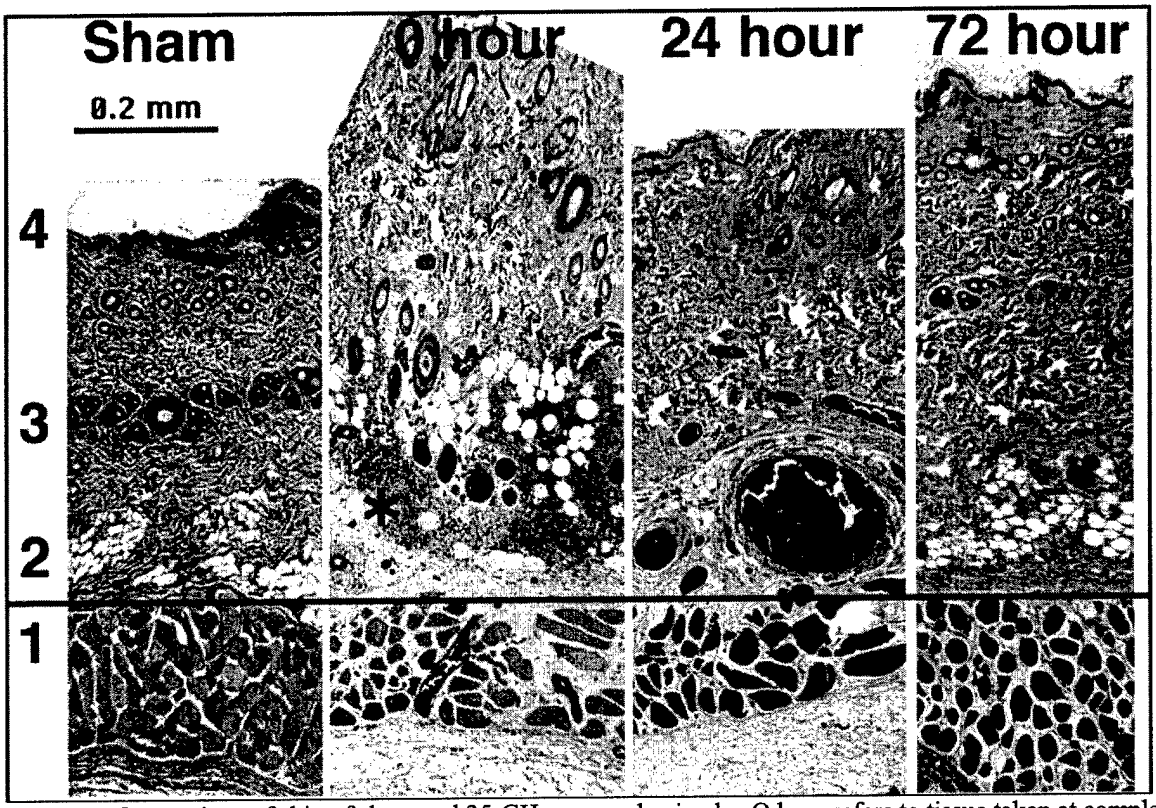


Figure 7: Comparison of skin of sham and 35-GHz exposed animals. O hour refers to tissue taken at completion of the 53 minute exposure; 24 hour refers to tissue taken 24 hours post-exposure; and 72 hour refers to tissue taken 72 hours post-exposure. Sham refers to an animal placed in exposure area for similar time to exposed animal and then taken for tissues (no exposure). Note bar scale under "Sham". All images at same magnification. Upper surface of striated muscle band for all samples adjusted to horizontal line. Numbers on left are coded as follows: 1 = muscle layer; 2 = adipose layer and primary vascular bed; 3 = mid dermis often with cut hair follicles; and 4 = epidermis. The asterisk in 0 hour indicates an extensive area of extravasated blood cells. Sections stained with H & E.

Tissues were also probed with Proliferating Cell Nuclear Antigen (PCNA). Images in Figure 8 reveal a PCNA response. With this type of probe the epithelium will generally show from a 10 to 25% positive response as the cells are performing general housekeeping. The injury resulting from prolonged MMW exposure was not uniform throughout. These MMW exposures produced

a "bullseye" type of response with the epithelial cells in the center being unresponsive and the cells on the perimeter being activated. Moving towards the periphery of the exposure zone, the epithelial cells return to a "housekeeping" mode again.

Immunohistological evaluation focused on the period 24 hours after exposure to MMW. Figure 8 shows that in the epidermis, proliferation is completely absent at the center of the exposed area, whereas relative hyperproliferation occurs at regions removed from the center by 2 cm. Similarly, Figure 9 reveals that significant oxidative stress occurs in structures immediately within the center of the exposed region and is relatively absent in regions slightly removed from the center of exposure. Figure 10 demonstrates that extensive leukocyte infiltration occurs following prolonged MMW exposure. Since there was clear evidence that many cells were dead or dying in various regions of the skin, we investigated whether apoptotic cell death was also triggered in seemingly unaffected cells (fibroblasts) 24 hours after exposure. Apoptotic cell death, as demonstrated by cytosolic expression of activated Caspase-3, was not evident anywhere in the dermis suggesting that cells that are committed to death are killed quickly.

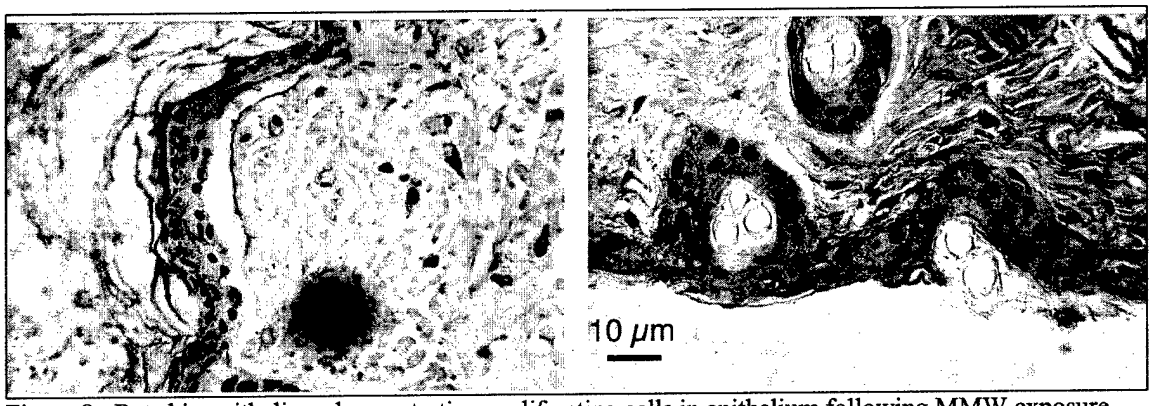


Figure 8: Rat skin epithelium demonstrating proliferating cells in epithelium following MMW exposure. Proliferative Cell Nuclear Antigen (PCNA) is represented by brown nuclear staining. Up-regulated PCNA indicates that cells are proliferating. These two images derived from the same tissue section are approximately 1 cm apart. The left image was near the center of the exposure area and the right image was near the edge of the exposure area and. The right image shows extensive proliferation of repair. In sham exposed skin, the percentage of positive cells is approximately 20%. Right hand image shows approximately 60% labeled indicating hyperproliferation. Complete absence of positive cells in left-hand panel suggesting that cells are no longer replicating.

We also probed the skin for expression of the enzyme inducible nitric oxide synthase (iNOS or NOS_2) and found that expression was somewhat reduced in the center of the exposed region though expression levels in controls were low (not shown). We hypothesized that expression may be important since NO is known to contribute to dermal repair following injury and is up-regulated in the plasma obtained from thermally-injured animals and humans.

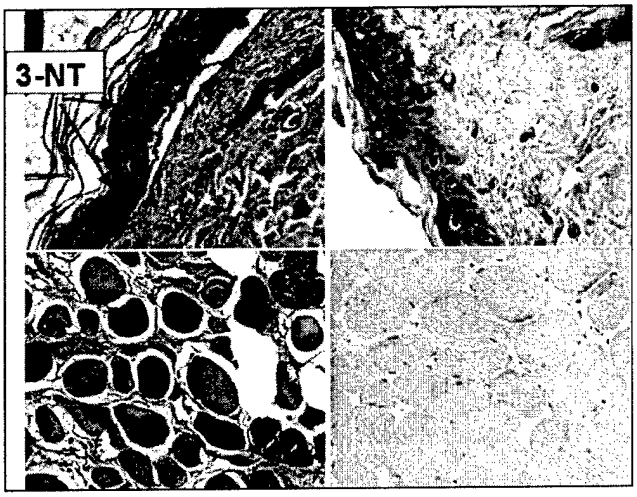


Figure 9: Oxidative stress in regions at the center of the exposure (left hand images) and 2 cm distant from the center. Upper images are the epidermis and the bottom images are the panniculus muscle. Brown staining indicates nitrated proteins. Proteins detected by immunohistochmical staining with polyclonal anti-nitrotyrosine specific antibody.

One of the most important findings is that the response of the dermis varies not only as a function of depth from the surface but also as a function of distance from the center of the exposure. This observation emphasizes the importance of performing in the future mathematical modeling of the distribution of energy through the skin during exposure.

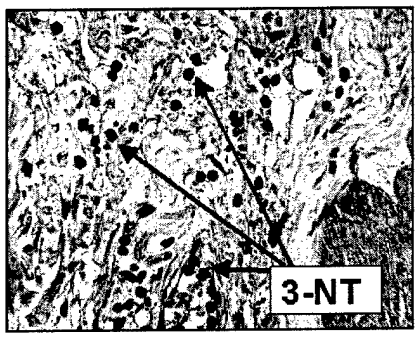


Figure 10: Leukocytes showing abundance of nitrated proteins 24 hr after MMW exposure.

Cytokines

Death could occur from a systemic up-regulation of inflammatory signaling molecules (e.g., cytokines TNFα, IL-1, IL-10) leading to injury in distant organs (e.g., lung, liver). Therefore, the levels of several cytokines (TNF-α, IL-1β, IL-6 IFN-γ and soluble phopholipase A-2 (sPLA2 - a rate-limiting enzyme of arachadonic acid pathway) in plasma were measured. IL-10 was not measured since it is not commercially available for rat tissue. The only significant change in these cytokines was in the concentration of IL-1β (see Figure 11).

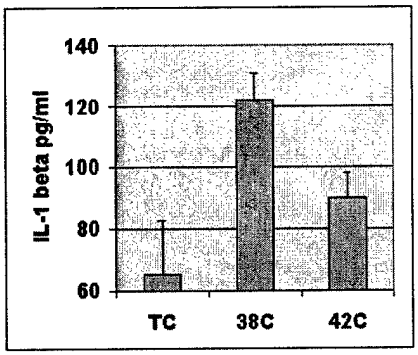


Figure 11: IL-1β in plasma during environmental heating.

Effects 24 Hours Following Exposure

An overexposed individual may not be able to reach a medical clinic within a short time after exposure. More than 24 hours may be required when at a remote worksite or battlefield environment. Thus, it is important that clinical biomarkers are capable of detecting biological changes several hours following exposure. At 24 hours post-exposure, we examined plasma and distant organs for evidence of oxidative stress and alterations in protein regulation. The results are shown in Figures 16 (plasma) and 17 (lung and skin).

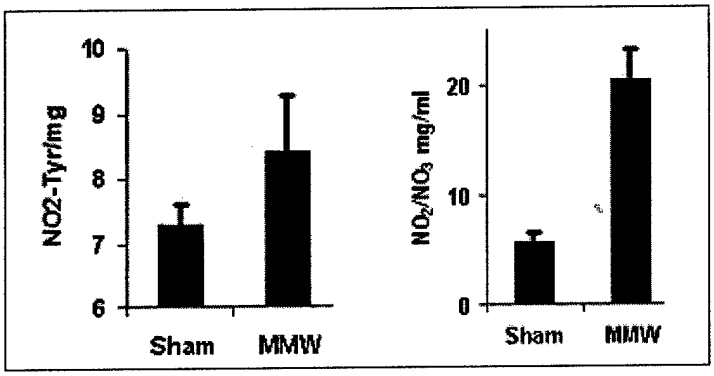


Figure 12: Nitrated proteins (NO_2) and NOx in plasma at 24 hour post-prolonged MMW

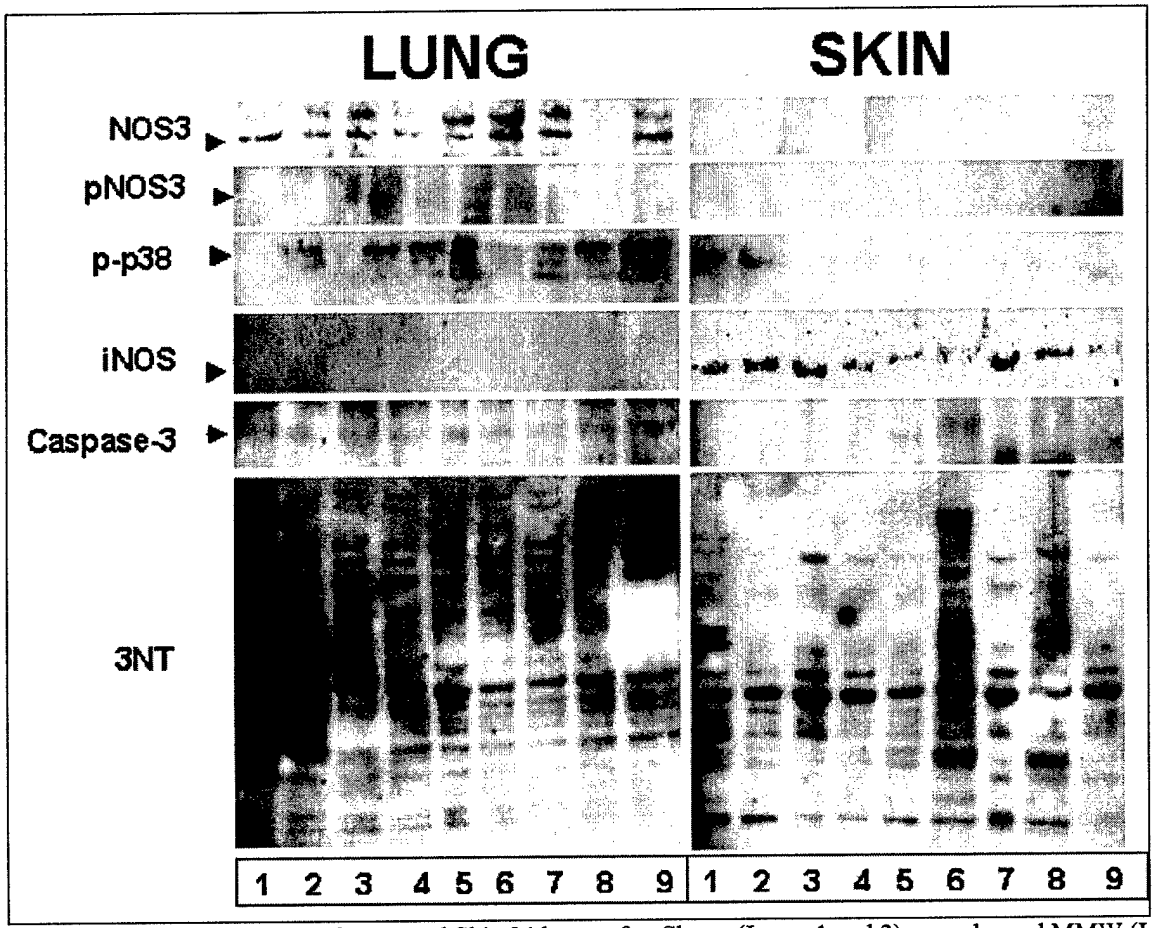


Figure 13: Western Blotting of Lung and Skin 24 hours after Sham- (Lanes 1 and 2) or prolonged MMW (Lanes 7-9) exposure.

Below are comments concerning the compounds represented in Figures 12 and 13.

(3-nitrotyrosine (3-NT) - Figure 12 shows that there is a distinct nitrated protein band found in skin of the MMW-exposed animals, but not in sham-exposed animal. This is in agreement with our immunohistochemical staining of tissue slices which shows that the epidermis and the muscle are heavily nitrated in exposed regions. Lung blot does not show evident changes in expression of nitrated proteins, though it is clear that they are abundant, confirming the results obtained from the ELISA.

Activated Caspase-3 (A protein that is associated with cellular commitment to apoptosis) - The blot reveals an increase an increase in levels of this protein following MMW exposure, but not after a sham exposure. This lends credence to concept signals transmitted from injured skin to distant organs, in this case the lung. The skin shows no evidence of this protein suggesting that apoptosis occurs before or after 24 hours or that necrosis predominates. This finding was also confirmed by immunohistochemistry (not shown).

Inducible nitric oxide synthase or iNOS (A protein that is important in the generation of NO following a wide range of insults and injuries) - During inflammation the enzyme is often upregulated. Skin is slightly NO positive with no clear expression pattern.

Phosphorylated p38 or pp38 (A member of an important family of early stress responsive signaling proteins) - Phosphorylated p38 levels increased in lung following MMW exposure, but were essentially undetectable in sham-exposed animals. This finding coincides with activated caspase-3 levels. In skin, levels in sham animals were higher than those in exposed animals suggesting that exposure pushes cells towards a stress pathway leading to the eventual loss of this phosphorylated form of this protein.

NOS_3 and p-NOS_3 (Phosphorylated (active) and non-phosphorylated forms of a constitutive form of nitric oxide synthase) - This protein is phosphorylated following a wide range of stimuli and may have an important role in nitration-based signaling. The blots do not indicate a clear pattern. Lung has high constitutive expression of NOS_3, which is not surprising given that this protein is constitutively expressed in vascular epithelium.

Survey Tools - Rat Alveolar Macrophages (ATCC designation NR 8388)

The cells used in this bioassay are rat alveolar macrophages (ATCC designation NR 8388). Lipopolysaccharide (LPS) is used as a positive control to validate that the cells respond to a well-known stimulus. The results of this bioassay are shown in Figure 14.

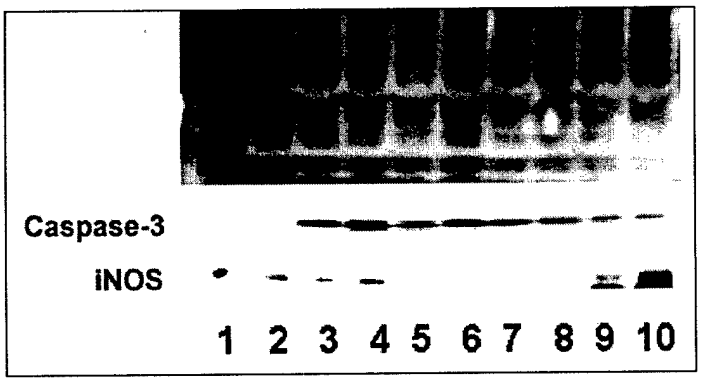


Figure 14: Effect of plasma from environmentally-heated rats on the activity of rat macrophage cell line *in vitro*. Cells exposed to 10% v/v plasma for 24 hours and then lysed. Treatments: 42°C (lanes 1 and 2); 38°C (lanes 3 and 4); plasma from sham-exposed rats (lanes 5 and 6); no plasma added (lanes 7 and 8); lipopolysacharide (LPS), 5 mg/ml, positive control (lanes 9 and 10). Protein loading is equal in each lane.

Caspase-3 (active form) indicates programmed cell death or apoptosis. The heavier bands indicate a higher percentage of cells undergoing apoptosis. As indicated in lanes 7 and 8, there

are a fraction of cells undergoing apoptosis at any given time, a finding that is not surprising for cells grown in culture. Cells stimulated with LPS have a smaller fraction undergoing apoptosis whereas cells exposed to plasma obtained from animals heated to 42°C do not go into apoptosis. As expected iNOS expression is heavily increased by LPS and by plasma obtained from heated rats, but not from sham-exposed rats. The nitrated protein Western blot showed that the heaviest nitration occurs in cells obtained from 42°C heated rats with very low levels occurring in LPS stimulated and non-stimulated cells. This would argue that protein nitration may protect cells from apoptosis. In order to further understand the role of NO and nitration chemistry we also determined the levels of NOx found in supernatants 24 hours after the addition of plasma. These results are found in Figure 15. Supernatants were also assayed for cytokines, however detectable levels of these were not observed (data not shown) except in LPS stimulated cells.

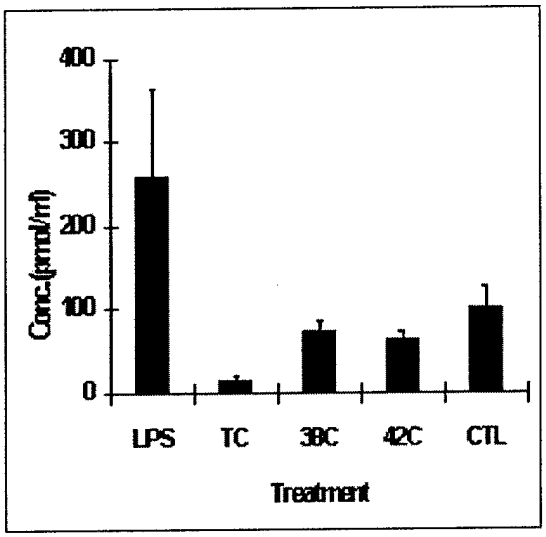


Figure 15: NOx production in rat alveolar macrophage 24 hours after addition of plasma. LPS = lipopolysaccharide; TC = plasma from sham rats; and CTL = no plasma added.

Survey Tools - Proteomics

Plasma samples were collected from rats exposed to MMWs or Environmental-Heat. These samples were sent to Dr. F. Witzmann for proteomic analysis. Tissues from the MMW-exposed rats were collected at three time points after exposure: 0, 24, and 72 hours. Tissue from the environmentally-heated rats were collected immediately after termination of exposure.

The following protein families appeared or were very much elevated after prolonged MMW exposure: alpha-1-acid-glycoprotein, T-kininogen I precursor, T-kininogen II precursor, haptoglobin alpha-chain, haptoglobin beta-chain, and possibly serine protease inhibitor 3. The appearance of these proteins is consistent with published literature showing an acute phase response following an intramuscular injection of turpentine into a rat (Hayes et al., 1998, Electrophoresis 19: 1484). Most importantly, these proteins do not appear in animals at the conclusion of MMW treatment (0 hour) (Figure 16C). At 24 hour post-treatment, the acute phase-like response is apparent and is very evident at 72 hour post-exposure (Figure 16D).

The data shown in Figure 16 demonstrates the promise that proteomics may have for the identification of substances that are uniquely associated with MMW exposure. These images suggest that as many as 10 proteins are produced in response to prolonged MMW exposure, but not during a turpentine insult. Clearly the goal of proposed research is to identify substances that

are produced during MMW exposure and not during control insults (e.g. environmental heating, infrared heating).

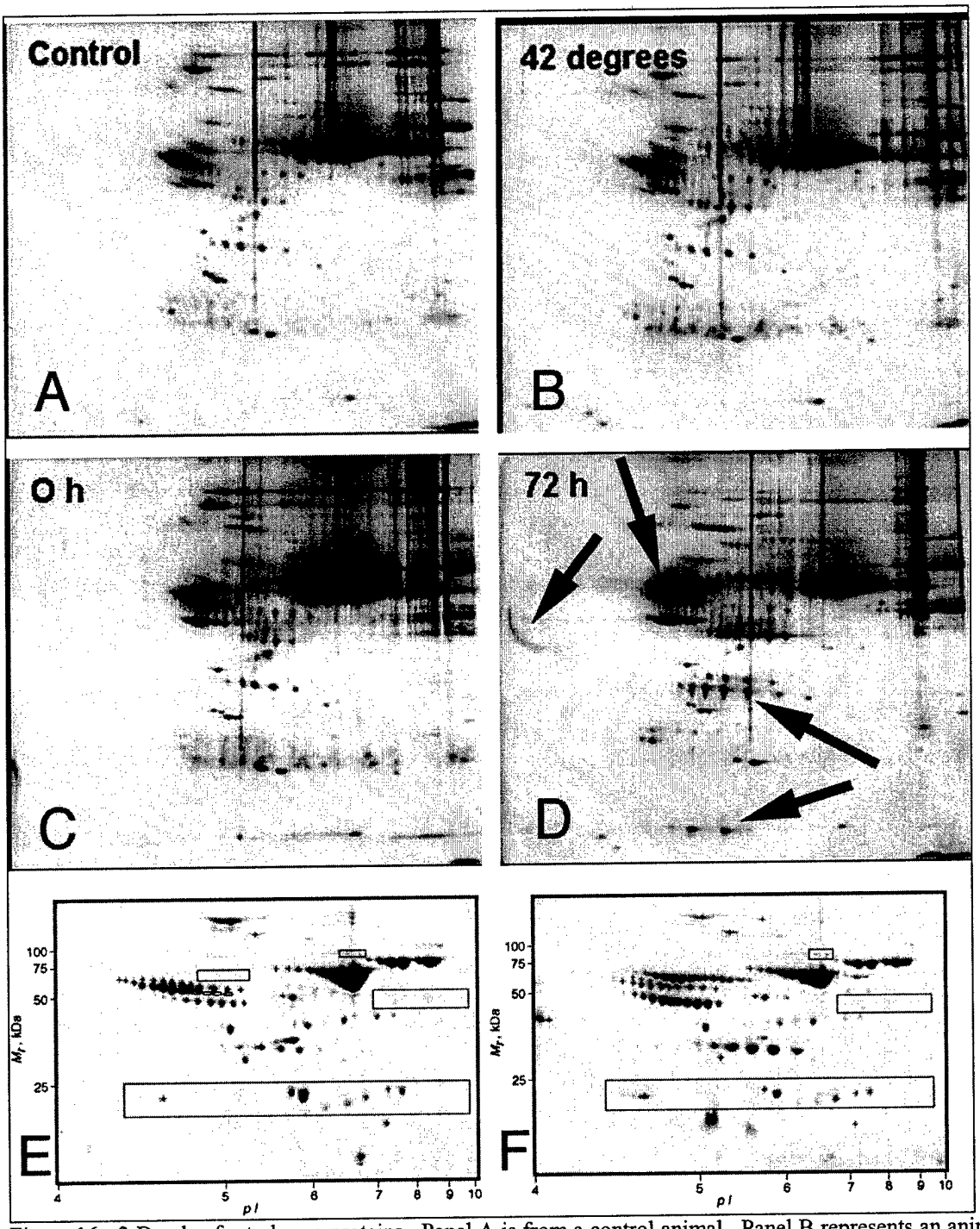
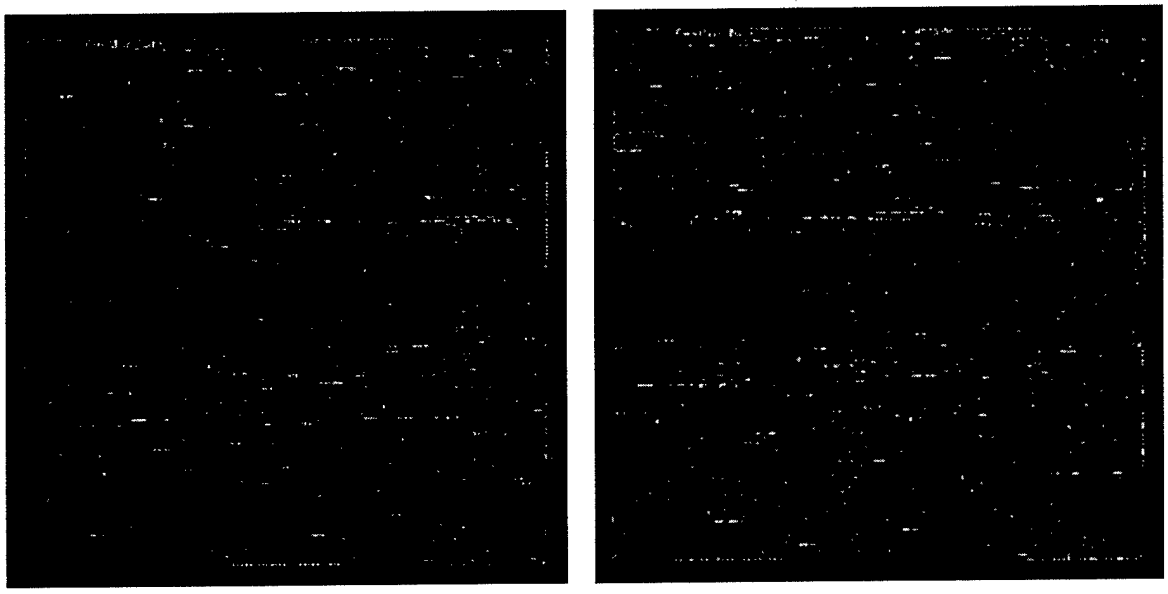


Figure 16: 2-D gels of rat plasma proteins. Panel A is from a control animal. Panel B represents an animal heated in warm ambient environment (42°C). Panel C is from an animal exposed to 35 GHz (90 mW/cm^2) and the blood sample was drawn immediately after exposure. Panel D is from an animal treated as in panel C, but the blood sample was drawn 72 hours post exposure. The arrows in panel D indicate points of difference with Control (A). Panels E and F were obtained from <http://linux.farma.unimi.it>. These are reference images for control rat serum (E) and from rats exposed to turpentine injected intramuscularly (F). Tentative points of interest in panel D were referenced to panel F.

Genomics Results

Three treatment groups of rats were used and include sham exposure, 35 GHz MMW prolonged exposure (50-60 minutes), and IR exposure. For IR, the exposure was adjusted so that the skin surface temperature profile matched that of the MMW exposure. Rats were allowed to recover and lung tissue was harvested 24hours after the end of exposure. Lung was selected because, as previously discussed, it is one of the organs that showed injury after systemic inflammatory events. RNA was extracted then submitted to Dr. John Frazier's laboratory at Wright Patterson AFB for gene expression analysis. For this analysis, RNA is hybridized to a rat genome gene array chip (Affymetrix GeneChip®) and fluorescently labeled. Each chip contains probes for 7000 different mRNA transcripts that correspond to genes for which the full-length sequence is known and code for proteins of known identity. The chip also contains probes for 1000 expressed sequence tag (EST) sequences. An EST sequence is a partial gene sequence and corresponds to a putative or as yet unidentified protein.

Figure 17 below shows the scanned images of the gene array chips for one sham and one MMW exposed animal. Fluorescence intensity of each spot indicates the amount of a particular mRNA present in the sample. The gene array images for MMW and IR exposed rats were compared to the image for the sham exposed rat. Thus, data represent the fold change in mRNA expression levels of exposed versus sham. The limit of sensitivity of this technology as reported by Affymetrix is a 2-3 fold change in mRNA levels.



SHAM **35 GHz MMW**

Figure 17: Scanned images of Affymetrix® rat genome gene array chips from analysis of lung tissue from sham and 35 GHz MMW exposed rats.

Initial mining of data from MMW exposed rats identified 28 genes or EST sequences for which changes in expression were detected. For IR exposed rats, changes in expression were detected in 12 genes. These genes are shown in table XX. The data represent changes that were detected in 2 rats for both exposure groups, i.e. duplicate gene chips were analyzed.

Several of the changes in gene expression noted above support the hypothesis of a thermal induced systemic immune response for MMW. This is indicated by the up-regulation of the pro-inflammatory mediator IL-1β as well as some other genes involved in inflammation that are briefly described below.

Myeloid-Related Protein 8 (MRP8) and MRP14: MRP8 and MRP14 are present in circulating neutrophils and monocytes and appear to translocate from the cytoplasm to the surface upon activation of the cell. For this reason, they are used as markers for activated or recruited phagocytes. At the surface, the proteins can form heterodimers and be released into the blood. MRP8 and MRP14 have been measured in the serum as a mean of monitoring inflammatory activity in patients suffering from rheumatoid arthritis, cystic fibrosis, and chronic bronchitis (Kerkoff *et al.*, 1999, Kerkoff *et al.*, 1998; Frosch *et al.*, 2000).

The exact functions of MRP8 and MRP14 are not completely understood. In vitro studies showed that both proteins are released from activated monocytes when cultured with TNF-stimulated endothelial cells (Frosch et al., 2000). MRP14 and MRP8 have been shown to be involved in adhesion and transendothelial migration of leukocytes by modulating the frunction of the neutrophil □$_2$ integrin CD11b (Newton and Hogg, 1998). Murine MRP8 is expressed in vascular endothelial cells after stimulation by IL-1 or TNF-α and it is thought to function as a chemotactic factor promoting leukocyte recruitment (Newton and Hogg, 1998).

Matrix Metalloproteinase (MMP-8): MMP-8 is also known as neutrophil collagenase and has been shown to play a major role in degradation of the extracellular matrix (ECM) during inflammatory diseases (Hanemaaiger *et al.*, 1997). Specifically, this enzyme degrades type I collagen. It is expressed in infiltrating monocytes and neutrophils in the lung and bronchial epithelial cells (Prikk et al., 2001). It is also produced in endothelial cells and fibroblasts in response to TNF-α. It is also produced in response to TNF-α (Hanemaaijer et al., 1997) and in human chondrocytes in response to IL-1, TNF-α, and IL-6 (Tetlow *et al.*, 2001). A recent study suggested that MMP-8 is activated from its precursor form by peroxynitrite or nitrogen dioxide radicals generated by activated neutrophils (Maeda *et al.*, 1998).

Major Acute Phase α-1 protein (MAP): This acute phase protein seems to be identical to T-kininogen, a precursor to a bradykinin analogue and therefore is thought to be involved in vasodilation, hypotension, increased capillary permeability, and nitric oxide formation by vascular endothelial cells (Yayama *et al.*, 2000). T-kininogen is expressed in vascular smooth muscle cells and fibroblasts in response to TNF-α, IL-1, and IL-6 (Takano *et al.*, 1995). *In vivo* data also shows that T-kininogen production is increased by TNF-α in rats (Utsunomiya *et al.*, 1998).

Plasminogen Activator Inhibitor-1 (PAI-1): Inflammatory related lung injury involves enhanced turnover and production of the ECM. PAI-1 is involved in this tissue remodeling by inhibition of plasmin, an action that leads to decreased degradation of ECM. An increased level of PAI-1 is thought to contribute to development of lung fibrosis. The source of PAI-1 appears to be endothelial cells, smooth muscle cells, activated macrophages and mast cells (Cho *et al.*, 2000).

Except for IL-1β, hypertension-regulated vascular factor-1C-4, and nuclear factor 1X, the genes that were either up- or down-regulated after MMW and infrared exposure are different. This could be due to differences in body core heating during exposure or the site of energy deposition within the skin. MMW penetrates deeper within the skin and causes core heating. Because this is just one time point, we can only draw limited conclusions at this time. Looking at gene

expression at an earlier time point and including environmentally-heated rats for comparison will add to this information.

MMW			IR		
Gene name	Fold change		Gene name	Fold change	
	Rat A	Rat B		Rat C	Rat D
Hypertension-regulated vascular factor-1C-4	11.5	8.2	Hypertension-regulated vascular factor-1C-4	11.1	7.1
Plasminogen activator inhibitor RNA-binding protein	8.5	18.8	Lipid binding protein	6.4	11.3
Myeloid-related protein-8	5.9	5.8	Plasminogen activator inhibitor-1	3.9	2.1
Myeloid-related protein-14	4.9	4.8	Interleukin 1-β	2.5	2.1
Matrix metalloproteinase-8	4.3	9.9	Carbonic anhydrase 5b	2.1	2.7
Interleukin 1-β	3.7	3.6	Mast cell carboxypeptidase A precursor	-4.6	-5.1
Carboxylesterase	3.6	2.8	Bone morphogenetic protein 3	-4.4	-3.2
Arginase	3.0	4.6	Receptor-linked protein tyrosine phosphatase -1	-3.7	-3.7
Heat shock protein 70	2.8	8.6	Transcription factor Oct1	-3.6	-3.4
Class IV alcohol dehydrogenase	2.6	3.0	Tenascin X	-3.3	-2.5
Major acute phase α-1 protein	2.5	2.6	RET ligand 2	-2.3	-2.3
MHC class II antigen RT1 B-1 beta chain	-30.7	-25.1	Nuclear factor 1-X	-2.2	-2.0
Stearoyl-CoA desaturase 2	-17.2	-25.7			
Taurine transporter	-11.3	-2.7			
Nuclear factor 1-X	-7.9	-4.3			
Ras-related rab1B protein	-7.0	-2.9			
Double-stranded RNA-specific editase	-6.1	-7.7			
Carboxypepetidase D precusor	-6.0	-2.1			
Latent TGF-β binding protein-2	-5.8	-7.5			
von Willebrand factor vWf precursor	-4.1	-6.8			
Protein kinase C epsilon	-3.9	-2.1			
β1-adrenergic recptor	-3.8	-5.0			
Fibronectin	-3.6	-2.7			
Signal transducing G protein alpha 12 subunit	-3.5	-2.4			
Sarcomeric mitochondrial creatine kinase	-3.3	-3.8			
Fibroblast growth factor receptor subtype 4	-2.8	-2.2			
Collagen α1 type 1	-2.2	-2.1			
Cytosolic retinol binding protein	-2.1	-2.2			

Table 1: Summary of genes for which changes in mRNA levels were detected in rat lung tissue using Affymetrix® rat genome gene array chips. mRNA from two rats exposed to MMW, Rats A and B, and from two rats exposed to IR, Rats C and D, were compared to mRNA from a sham exposed rat. Lung tissue was harvested at 24 hours post-exposure.

Time to Death Study

After a variety of efforts to determine how best to expose animals, the time to death study was conceived. Animals were given a continuous exposure of MMW until they were pronounced dead. This response would then become the benchmark for LD100 (lethal dose 100% of the time). It would also allow direct comparison between exposure types. Figure 18 provides the graphic evidence of this necessary study.

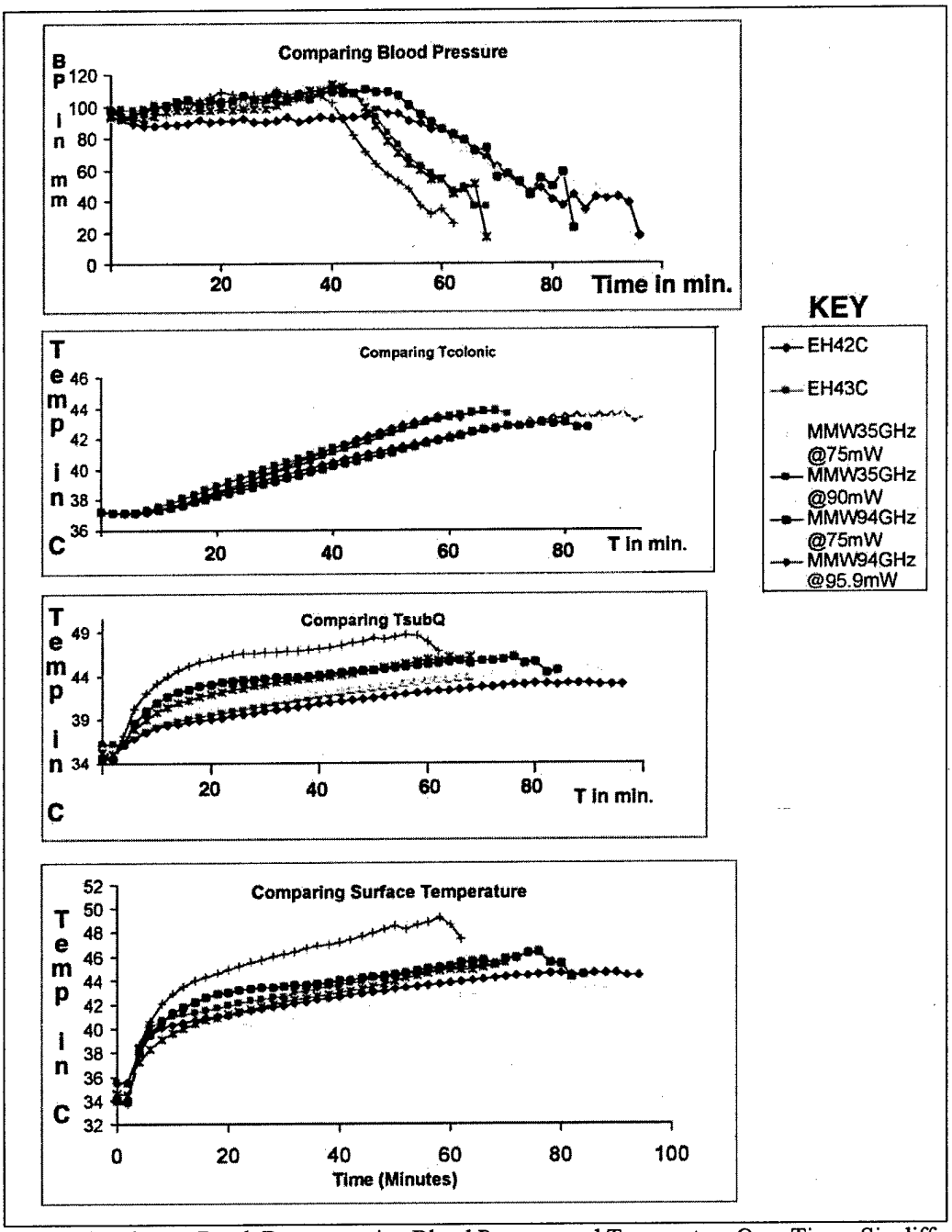


Figure 18: Time to Death Demonstrating Blood Pressure and Temperature Over Time. Six different exposure conditions are represented: EH42°C, EH43°C, 35GHz@75mW, 35 GHz@90mW, 94GHz@75mW, and 94GHz@95.9mW. n=6 for each exposure condition.

As can be seen from the data on the second graph "Comparing Tcolonic", the core body temperature profiles matched. The EH42C, 35GHz@75mW, and 94GHz@95.9mW matched well. The EH43C, 35GHz@90mW, and 94GHz@95.9mW matched each other well. The two groups of exposures have their respective slopes. When one compares the Blood Pressure data (top graph in the figure) with these two exposure groups in mind, the difference between the two groups continues. The stronger exposure group (known as the 43C group) demonstrates that animals were succumbing nearly twenty minutes sooner than the less strong exposure group

(known as the 42C group). This difference is more graphically seen in the top panel in Figure 19. Pathology indicated a higher probability of burn morphology with the 43C group. The end colonic temperature values were within 0.7°C between each of the six exposure groups. (see second panel on Figure 19) The TsubQ (data from a subcutaneous temperature probe) and Surface Temperature measurements continue the trend to cluster groups 42C and 43C temperature elevations. Clearly the 94GHz@95.9mW showed a more rapid rise in surface and subQ temperature response and a more rapid decline in blood pressure. The most striking difference in response is represented in the bottom panel of Figure 19. The end surface temperature response of the 94GHz@95.9mW treatment at death was five degrees higher than the other five exposure regimens. Based on these collective findings we oriented our work towards the 42C group exposure. (Please note that each exposure group in this Time to Death study is based on a "n" of 6. As of May 2002 the statistical analysis of the Time to Death data had just started.)

As future work moves towards using recovery animals for genomic and proteomic investigation, the 42C group exposure regimen becomes increasingly important. We will need exposures from LD25% and lower to produce animals suitable for recovery-based studies. Also important is that the biomarkers we seek will involve individuals who have no overt signs of exposure. A systematic search for threshold response should be the next step in this study. Additionally, with core body temperature elevation so consistent between exposure regimens, we will focus on adjusting the time of exposure to the core body temperature profiles.

The Time to Death study when coupled to pathology observations of the tissue suggests that where the energy deposits in the tissue may be different between different exposures. The histology for 35GHz is shown in Figure 7. Not shown here is the data for 94GHz. It appears that both the 35GHz and 94GHz exposures heat each the tissue from the inside out in contrast with EH and IR treated tissue. There also appears to be differences between 94 and 35 GHz treated tissues. At the same power density the 94GHz exposed animals seem more damaged. This phenomenon should be followed in greater detail.

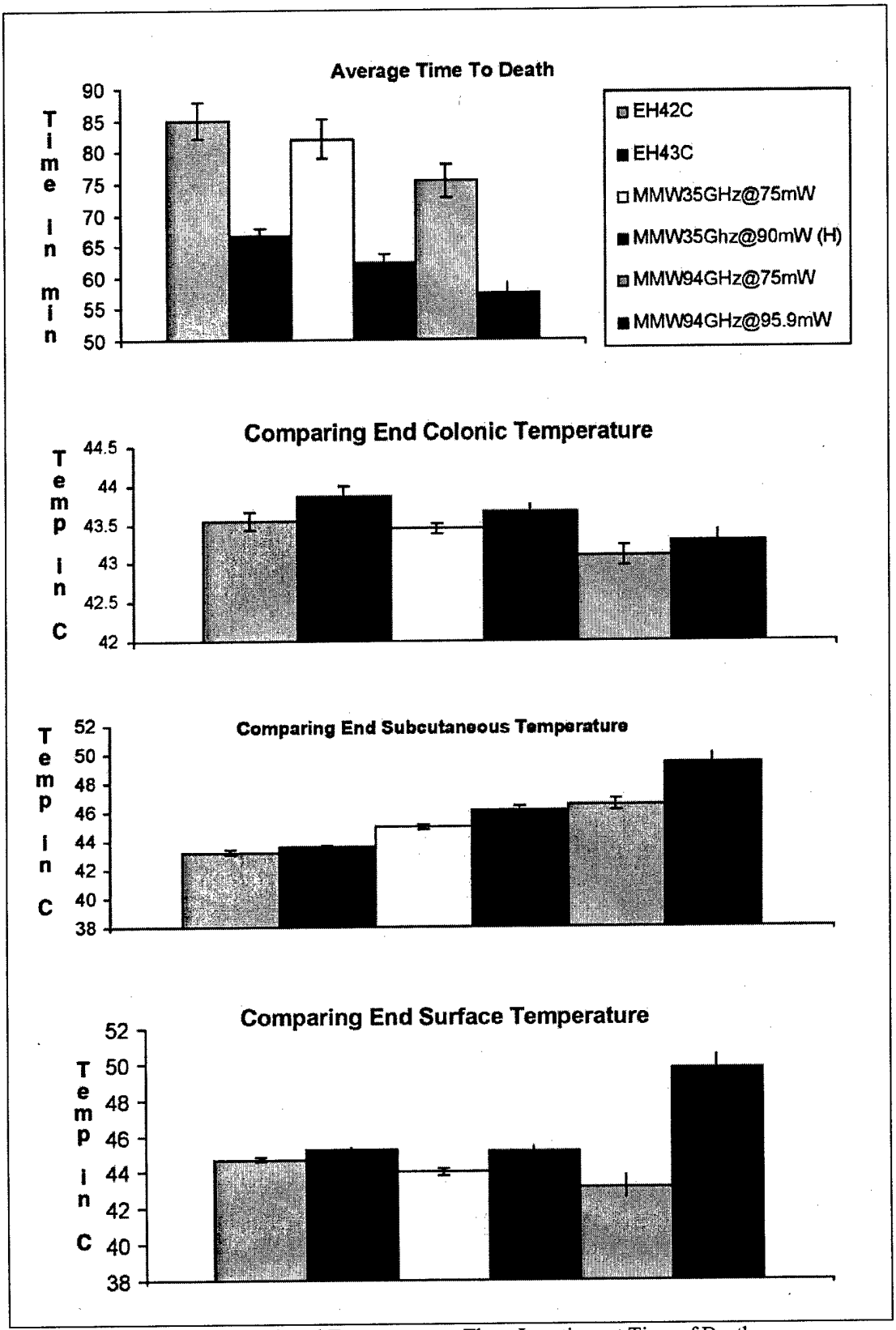


Figure 19: Mean Time to Death and Temperature at Three Locations at Time of Death.

The Objectives of the Proposal

The research grant as originally proposed was based on five objectives that are listed below and very briefly the relevant progress towards those objectives.

Objective 1 - Find relevant biomarkers of MMW exposure.

Progress has been made in this area. We now have several candidates for future investigation.

Objective 2 – Determine relevancy of MMW biomarkers at other frequencies.

Progress has been made is the area. We have compared 35 GHz (the original target) to 94 GHz. There appears to be differences in animal response between these two frequencies.

Objective 3 – Determine specificity to animal strain and species.

This objective was not explored due to a lack of time and that our findings suggested that there might not be disagreement with other species.

Objective 4 – Determine how biomarker expression from RFR exposure compares to that from other environmental stressors.

We have compared RFR to Environmental Heat at several levels and to Infrared at several levels. EH and IR both heat from the outside and the MMW from the inside. Strict comparisons are difficult although IR may be unsuitable.

Objective 5 - Determine the effects of pharmacological agents on biomarkers of RFR exposure.

Some effort was made here but time and the lack a specific biomarker did not allow much progress.

RESULTING PUBLICATIONS AND PRESENTATIONS

Kalns, J., K.L. Ryan, P.A. Mason, J.G. Bruno, R. Gooden, and J.L. Kiel. Oxidative stress precedes circulatory failure induced by 35-GHz microwave heating. *Shock*, 13: 52-59, 2000.

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